Seasonal variations in optimized applications of intermediate temperature stable α-amylase in raw sugar manufacture †

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abstract

In recent years, starch being delivered to and processed in U.S. factories has risen markedly because of the increased production of green (unburnt) and combine-harvested (billeted) sugarcane as well as the introduction of new sugarcane varieties with higher starch content. This has led to warnings by some U.S. refineries that there may be a penalty for high starch concentrations in raw sugar if starch control is not improved. To prevent carry-over α-amylase activity in molasses and raw sugar in the U.S., commercial α-amylases used to control starch are intermediate temperature (IT) stable and sourced from *Bacillus subtilis* bacteria. α-Amylases have been typically applied to syrup in last evaporators where starch is solubilized and gelatinized, syrup temperatures are ~60-65°C, and ~18 min retention time (R_t) is available. As IT stable α-amylases are effective up to 85°C, they could be more effective and economical if applied to next-to-the-last evaporators where syrup temperatures are ~77°C. Factory α-amylase trials were conducted across the 2007 Louisiana processing season (Oct-Dec). Application of a working solution (diluted 3-fold in water at the factory) of IT stable α-amylase of high activity per unit cost (118.3 KNU/ml/$) to the next-to-the-last evaporator provided significantly (P<.05) greater starch hydrolysis (up to 78.0% at a 5 ppm/cane wt dose) than applying it to the last evaporator alone (only up to 59.8% at a 5 ppm/cane wt dose). Reasons for the improved starch hydrolysis in the next-to-the-last than the last evaporator are multi-fold: (i) the lower Brix levels in the next-to-the last evaporator improve α-amylase action, (ii) more water is available as a reactant for the hydrolysis reaction, and (iii) there is more time for the hydrolysis reaction to occur. Starch hydrolysis, generally, increased polynomially with increasing initial concentrations of starch in syrups. Seasonal variations in starch concentrations affected the application of α-amylase to next-to-the-last evaporator more than to the last evaporator alone. Significantly (P<.05) less starch was hydrolyzed with lower precision when starch concentrations were <1000 ppm/Brix in late season (Dec), because of lower contact between the starch and α-amylase. Fluctuating starch concentrations across the season make standardized application of α-amylase impossible. Final recommendations are provided.

Keywords: **α-amylase, *Bacillus subtilis* seasonal starch variations, next-to-the-last evaporators, raw sugar manufacture**

Variaciones estacionales en las aplicaciones optimizadas de la α-amilasa, estable a temperaturas intermedias, en la manufactura de azúcar crudo

En los últimos años, el almidón suministrado y para ser procesado en plantas de los EEUU ha aumentado considerablemente debido al incremento de la producción de caña de azúcar verde (sin quema) y cosechas con segadoras/trilladoras (trozado), así como a la introducción de variedades de caña de azúcar con alto contenido de almidón. Esto ha hecho que algunas refinerías de los EEUU advirtieran que podrían aplicar multas frente a la alta concentración de almidón en el azúcar crudo si el control del mismo no mejoraba. Para prevenir el arrastre de actividad de α-amilasa en la melaza y en el azúcar crudo en los EEUU. Las α-amilasas comerciales utilizadas para controlar el almidón son estables a temperatura intermedia (IT) y provienen de la bacteria *Bacillus subtilis*. Las α-amilasas se han añadido habitualmente al jarabe en los últimos evaporadores, en los que el almidón se solubiliza y gelatiniza, las temperaturas del jarabe son de ~60-65°C y se dispone de un tiempo de retención (Rt) de ~18 m. Como las α-amilasas estables a IT son efectivas hasta los 85°C podrían ser mas efectivas y económicas si se aplicaran al anteúltimo evaporador, cuando las temperaturas son de ~77°C. En Louisiana, a lo largo de la época de producción de 2007 (octubre a diciembre), se llevaron a cabo pruebas de planta con la α-amilasa. La utilización de una solución de trabajo (diluida a 1/3 con agua en la fábrica) de α-amilasa IT estable de alta actividad por unidad de costo (118,3 KNU/ml/$) al anteúltimo evaporador produjo una hidrólisis significativamente mayor (P<0,5) (hasta un 78% a una dosis de 5ppm/caña en peso) que su aplicación al último evaporador solamente (solo hasta el 59.8% a una dosis de 5ppm/caña en peso). Las razones para una hidrolisis del almidón mayor en el anteúltimo evaporador que en el último son múltiples: (i) los menores niveles de Brix en el anteúltimo evaporador mejoran la acción de la α-amilasa, (ii) se dispone de más agua como reactivo para que se produzca la reacción de hidrólisis, y (iii) se dispone de más tiempo para que se produzca la hidrólisis. La hidrólisis del almidón, en general, se incrementa polinómicamente con el aumento de la concentración inicial de almidón en los jarabes. Las variaciones estacionales en la concentración de almidón afectan la aplicación de la α-amilasa al anteúltimo evaporador más que la aplicación sólo al último. Cuando las concentraciones de almidón fueron <1000 ppm/Brix al final de la estación (Diciembre) se hidrolizó significativamente (P<0,5) menos almidón y con menos precisión, debido al menor contacto entre el almidón y la α-amilasa.

Las fluctuaciones en la concentración de almidón a lo largo de la temporada hacen imposible la aplicación estandarizada de la α-amilasa. Se incorporan recomendaciones finales.

Saisonale Schwankungen in der optimierten Beigabe von Alpha-Amylase mittlerer Temperaturbeständigkeit bei der Rohzuckerherstellung

In den letzten Jahren hat die Menge der Stärke, die an US-Fabriken geliefert und von diesen verarbeitet wird, erheblich zugenommen. Dies liegt sowohl daran, dass mehr grünes (unverbranntes) und mit Mähdreschern geerntetes (gehacktes) Zuckerrohr produziert wird, als auch daran, dass neue Zuckerrohrarten mit höherem Stärkegehalt eingeführt worden sind. Dies hat zu Warnungen einiger US-Raffinerien geführt, dass, falls die Stärkekontrolle nicht verbessert werde, die hohen Stärkekonzentrationen im Rohzucker zu Nachteilen führen könnten. Um in den USA die Übertragung von Alpha-Amylase-Akvitität in Melasse und Rohzucker zu verhüten, werden zur Stärkekontrolle Alpha-Amylasen eingesetzt, die von mittlerer Temperaturbeständigkeit (IT-stabil) und aus *Bacillus subtilis* Bakterien gewonnen sind. Alpha-Amylasen sind dem Sirup bisher üblicherweise in den letzten Verdampfern beigegeben worden, in denen die Stärke gelöst und geliert wird, die Siruptemperatur bei ~60-65°C liegt und eine Mindestverweildauer (Rt) von ~18 Min. gegeben ist. Da IT-stabile Alpha-Amylasen bei bis zu 85° C wirksam sind, könnte es wirksamer und wirtschaftlicher sein, wenn sie den vorletzten Verdampfern beigegeben würden, in denen eine Siruptemperatur von ~77°C herrscht. Fabriksversuche mit Alpha-Amylasen wurden während der Louisiana Verarbeitungssaison (Okt. – Dez.) 2007 durchgeführt. Die Beigabe einer Arbeitslösung (in der Fabrik dreifach in Wasser verdünnt) von IT-stabiler Alpha-Amylase hoher Aktivität pro Kosteneinheit (118,3 KNU/ml/$) zum vorletzten Verdampfer lieferte eine signifikant höhere (P<,05) Stärkehydrolyse (bis zu 78,0 % bei einer Dosis von 5 ppm/Zuckerrohrgewicht) als deren alleinige Beigabe zum letzten Verdampfer (nur bis zu 59,8 % bei einer Dosis von 5 ppm/Zuckerrohrgewicht). Die Gründe der verbesserten Stärkehydrolyse im vorletzten anstatt letzten Verdampfer sind verschiedener Art: (i) die niedrigeren Brix-Level im vorletzten Verdampfer verbessern die Alpha-Amylase-Aktion, (ii) es steht mehr Wasser als Reaktand für die Hydrolyse-Reaktion zur Verfügung, und (iii) es gibt mehr Zeit, in der die Hydrolyse-Reaktion erfolgen kann. Die Stärkehydrolyse nahm bei zunehmenden Eingangskonzentrationen von Stärke in Sirupen im Allgemeinen polynomiell zu. Saisonale Schwankungen in den Stärkekonzentrationen beeinflussten die Beigabe von Alpha-Amylase zum vorletzten Verdampfer mehr als nur zum letzten Verdampfer. Signifikant weniger (P<,05) Stärke wurde mit niedrigerer Präzision hydrolisiert, wenn die Stärkekonzentrationen in der Spätsaison (Dez.) <1000 ppm/Brix waren, weil ein geringerer Kontakt zwischen Stärke und Alpha-Amylase gegeben war. Fluktuierende Stärkekonzentrationen während der Saison machen standardisierte Zugaben von Alpha-Amylase unmöglich. Abschießende Empfehlungen werden gegeben.

As variações sazonais em aplicações otimizadas de α-amilase estável em temperatura intermediária na fabricação de açúcar bruto

Nos últimos anos, o amido entregue e processado nas fábricas dos EUA aumentou significativamente devido ao aumento da produção de cana-de-açúcar verde (não queimada) e de colheita combinada (armazenada) de cana, bem como a introdução de novas variedades de cana com maior teor de amido. Isto levou a advertência por parte de algumas refinarias norte-americanas de que penalidades poderiam ser impostas para as concentrações de amido em açúcar bruto se o controle não for melhorado. Para prevenir a continuação da atividade da α-amilase no melaço e no açúcar bruto comercial nos Estados Unidos, as α-amilases comerciais usadas para controle de amido são estáveis em temperatura intermediária (TI) e originária da bactéria *Bacillus subtilis*. As α-amilases são normalmente usadas no caldo nos últimos evaporadores onde o amido está dissolvido e gelatinizado, a temperatura do caldo está entre ~60-65°C, e o tempo de retenção (Rt) de ~18 está disponível. Como as α-amilases estáveis em TI são efetivas até a temperatura de 85°C, elas poderiam ser mais efetivas e econômicas se utilizadas nos penúltimos evaporadores onde a temperatura do caldo é de ~77°C. Experimentos em fábrica com a α-amilase foram realizados durante a temporada de processamento de Louisiana em 2007 (Out-Dez). A aplicação de uma solução ativa (diluída em 3 partes de água na fábrica) de α-amilase estável em TI de alta atividade por custo de unidade (118,3 KNU/ml/$) no penúltimo evaporador resultou em uma hidrólise de amido significativamente (P<0,05) maior (até 78% a uma dose de 5 ppm/cana wt) do que a utilização no último evaporador (até 59,8% a uma dose de 5 ppm/cana wt). As razões para uma melhor hidrólise do amido no penúltimo evaporador em comparação com o último são: (i) os níveis mais baixos de Brix no penúltimo evaporador melhora a ação da α-amilase, (ii) mais água está disponível como reagente para a reação de hidrólise, e (iii) há mais tempo para a reação de hidrólise acontecer. A Hidrólise de amido, geralmente, aumenta de forma polinomial com o aumento das concentrações iniciais de amido nos caldos. As variações sazonais nas concentrações de amido afetaram a utilização da α-amilase no penúltimo evaporador mais do que no último evaporador sozinho. Expressivamente menos (P<0,05) amido foi hidrolisado com baixa precisão quando as concentrações de amido eram <1000 ppm/Brix no final da temporada (Dez), devido ao menor contato entre o amido e a α-amilase. As concentrações flutuantes de amido durante a temporada tornam impossível a normatização da utilização da α-amilase. Foram fornecidas recomendações finais.

Introduction

Starch being delivered to and processed at U.S. factories has risen markedly in recent years because of the increased production of green (unburnt) and combine-harvested (billeted) sugarcane (Godshall *et al*, 2005) as well as the introduction of newer varieties with high starch content (Eggleston *et al*, 2010). Starch is an undesirable impurity because it causes processing difficulties in the factory and refinery (especially a carbonatation refinery) (Eggleston *et al*, 2007a, 2008). For these reasons, U.S. factories are being encouraged to deliver raw sugar containing <250 ppm/Brix starch, with <200 ppm/Brix preferred to a Louisiana (LA) carbonatation refinery (F. Goodrow, Domino Sugar, personal communication). In comparison to other countries, there is no current penalty in the U.S. for high starch concentrations in raw sugar (Eggleston *et al*, 2008).

Starch, produced in the sugarcane plant exists as a semi-crystalline granule that contains two glucose polysaccharides: ~19% amylose and 81% amylopectin, and is extracted into juice on factory tandem milling or diffusion. Amylose is linear with the glucose molecules α-D-(1→4) linked. Amylopectin, in addition to the α-D-(1→4)-linked glucose found in amylose, also contains many α-D-(1→6)-linked branch points. Starch concentrations in a particular factory or refinery product vary widely depending on

country, season, variety, amount of leaves and tops processed, diurnal cycle, sugarcane disease, sugarcane maturity, processing conditions, effectiveness of removal techniques, and method of analysis.

α-Amylase (endo-1→4-α-D-glucan glucohydrolase; EC 3.2.1.1) hydrolyzes solubilized and gelatinized starch much faster than granular starch because starch crystallinity is lost and there is greater accessibility of the α-amylase to the exposed polysaccharides (Tester *et al*, 2004). Starch is solubilized and gelatinized during diffusion, clarification, and evaporation (Eggleston *et al*, 2008). α-Amylases in the presence of water, randomly hydrolyze or cleave 1→4-α-D-glycosidic linkages between adjacent glucose molecules in the amylose chain. The viscous solution is progressively "thinned" into lower MW dextrins, and finally maltodextrins (oligosaccharides) of smaller chains (often in the 2-7 dp range) that are more manageable. The full characterization of commercial α-amylases used in the U.S., including their effective temperature and pH ranges were recently reported by Eggleston *et al* (2008). α-Amylases used in the U.S. exist in a wide range of activities (59.0 to 545.3 KNU/ml) that do not reflect their comparative unit costs (Eggleston *et al*, 2008). This problem is exacerbated in the sugar industry by there being no uniform or standard method to measure the α-amylase activity (Note: factory α-amylase activity methods are available but they have not been standardized for the sugar industry). Presently, there is also the concern in the sugar industry about the use of α-amylases from *Bacillus licheniformis* and *stearothermophilus* that were specifically engineered to have extreme high temperature (HT) stability (up to 115°C) for applications in larger markets other than the sugar industry, e.g., detergents (Eggleston and Montes, 2009). Such HT α-amylases can be too temperature stable in the sugar industry, and may not denature/inactivate after application, resulting in carry-over activity into raw sugar and molasses. α-Amylase activity in the raw sugar can even carry through subsequent refinery processes and eventually reside in refined sugar, molasses, and food products (Eggleston and Montes, 2009). To avoid this, large customers of U.S. refineries have requested that HT stable and any other α-amylases are not applied at the refinery (F. Goodrow, Domino Sugar, personal communication). Concomitantly, U.S. refineries have requested factories not to apply HT stable α-amylases (F. Goodrow, Domino Sugar, personal communication). Because of the recent increase of starch concentrations in products across U.S. sugarcane factories, there have been warnings by some U.S. refineries that there may be a penalty for high starch concentrations in raw sugar if starch control is not improved. Thus, we conducted a series of trials to improve and optimize the factory α-amylase application of *Bacillus subtilis* α-amylases which are intermediate temperature (IT) stable and do not have carry over problems (Eggleston *et al*, 2007a, 2008).

In the U.S. and many other countries, α-amylases have been most commonly applied to factory last evaporators where syrup temperatures are ~60-65°C (140-149°F) and starch is solubilized/gelatinized. α-Amylase application to diffusers has been studied (Schoones *et al*, 2006) but was not technically feasible mostly because the juice is >92°C. In our first trials (Eggleston *et al*, 2007a) we applied IT stable α-amylases to syrup in the last evaporators of three LA sugarcane factories The traditional application of IT stable *B. subtilis* α-amylase with low activity (59 KNU/ml) gave disappointing and uneconomical starch hydrolysis results. In contrast, the application of a higher activity (545.3 KNU/ml) IT stable α-amylase gave improved results although problems still existed because of (i) low contact between α-amylase and starch and (ii) the low water activity of syrup. Fortunately, application of the high activity IT stable α-amylase as a working solution, first diluted 3-fold in water at the factory, improved starch hydrolysis (but still only up to 42%) and was more cost-effective than adding the α-amylase undiluted (Eggleston *et al*, 2007a). As IT stable α-amylases are effective up to 85°C (185°F) (optimum is ~70°C; Eggleston *et al*, 2008), they may be even more effective and economical if applied to next-to-the-last (penultimate) evaporator where syrup temperatures are ~77°C (170°F). Preliminary results from a 2006 trial at a LA factory, where a 3-fold working solution of high activity IT stable α-amylase was applied to the next-to-the-last evaporator and the last evaporator suggested more starch was hydrolyzed than when applied in the last evaporator alone, and warranted further investigation. This paper reports results from factory trials undertaken to compare the application of IT stable α-amylase to the next-to-the-last evaporator compared to the last evaporator alone across the 2007 LA processing season, to cover varying physiological states of the sugarcane and environmental conditions.

Materials and methods

α-Amylase activity

The activity of α-amylases used in LA factories was based on the PhadebasTM method of Novozyme (Anon, 2001), with major modifications. The method was conducted at 65°C and pH 6.4 to simulate conditions in last evaporators. Phadebas™ tablets (Pharmacia Diagnostics, U.S.), composed of cross-linked, insoluble, blue color starch polymer, were dissolved in distilled water and the starch hydrolyzed with applied α-amylase to give blue fragments. Absorbance of the resulting blue solution at 620 nm is proportional to the α-amylase activity. A standard curve was first produced based on Termamyl™ α-amylase (*Bacillus licheniformis*) of known activity (120 KNU/ml).

Brix

The mean Brix of triplicate samples was measured using an Index Instruments TCR 15-30 temperature controlled refractometer accurate to ± 0.01 Brix.

Starch

Starch was measured using the rapid SPRI iodometric method (Godshall *et al*, 2004) with modifications (Eggleston *et al*, 2007a). Because of the large variability in the Brix's of the different syrups collected, their dilutions in de-ionized water differed to give final Brix values of ~15.0. For syrups entering the next-to-the-last (A_{2in}) evaporator, ~50 g syrup was first dissolved in 50 ml water. For syrups entering the next-to-last (A_{2out}) evaporator, ~37 g syrup was dissolved in 63 ml water. For syrups exiting the last (A_{3out}) evaporator ~23 g syrup were dissolved in 77 ml water. The

Figure 1a. Configuration of Robert's-type evaporators at the Louisiana sugarcane factory under study. First day sampling. Letters denote the set of evaporators. Numbers denote when sampling occured taking into account retention time. The average Brix of the juice entering A_2 evaporator was 24, exiting A_2 was 38, and exiting A_3 evaporator was 65

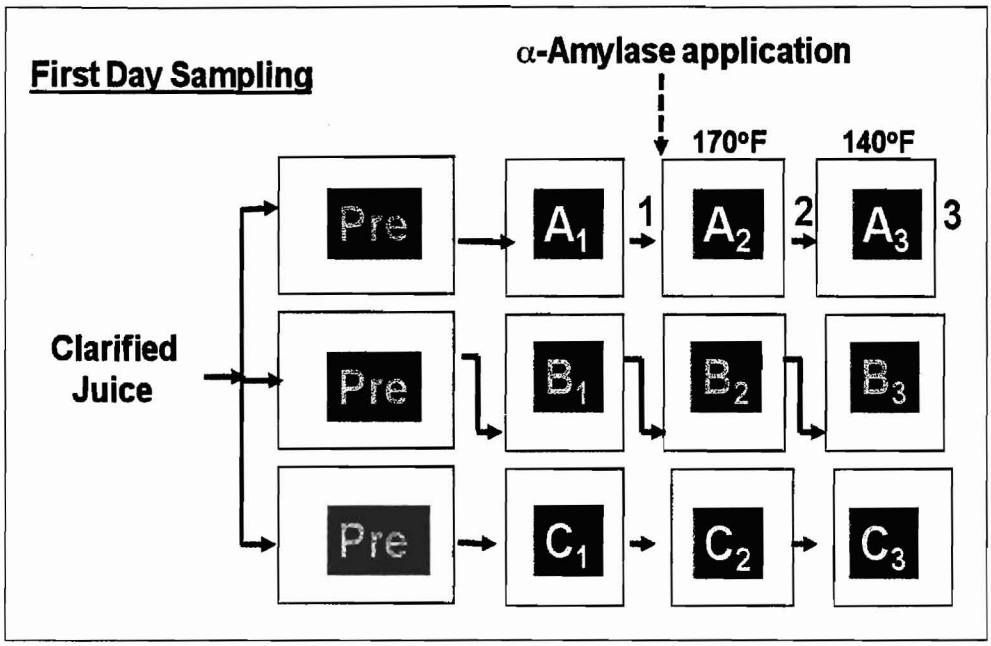
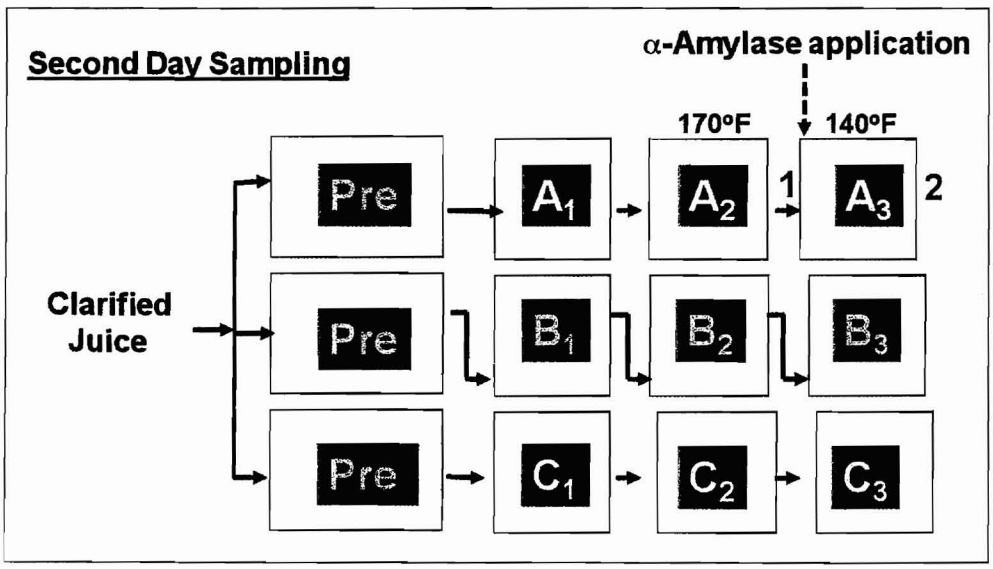


Figure 1b. Configuration of Robert's-type evaporators at the LA sugarcane factory under study. Second day sampling. Letters denote the set of evaporators. Numbers denote when sampling occured taking into account retention time. The average Brix of the juice exiting A_2 was 38, and exiting A_3 evaporator was 65



syrup solution was first boiled for 5 min to completely solubilize starch. Formed blue/purple specific starch-iodine complex was measured at 600 nm. Starch was assayed in duplicate samples and concentrations are quoted as average ppm/Brix.

General factory sampling

The LA factory capacity was ~13,700 short tons cane/day and the factory operated a tandem mill. Syrups before and after the application of α-amylase in the factory were collected in containers (250 ml) with 5 drops of biocide (Bussan 881™, Buckman Labs., U.S.), then immediately placed in ice before transportation to the laboratory and stored in a -40°C laboratory freezer prior to analyses. Before all trials, factory α-amylase applications were stopped, and the existing syrup in the evaporators purged for 1-2 h to equilibriate the system.

α-Amylase factory trial

The factory operated a quadruple evaporator station (three sets denoted A, B, and C) composed of Robert's-type evaporators (Figure 1), and only Set A was used in this study. Prior to this study, α-amylase was typically applied with a Solenoid Driven Metering Pump (6 L/hour maximum capacity; Tacmina Corp., Japan) directly into the last evaporator body with syrup R_t of 18 min (Eggleston and Monge, 2005), syrup temperature ~ 50°C (122°F) and the Brix of exiting syrup ~65. In this study, α-amylase was also applied into a specially installed inlet pipe of the next-to-the last evaporator (Figure 1a) where syrup R_t was 11 min, syrup temperature ~77°C (170°F) and av. Brix of exiting syrup was 38.

The first day of sampling occurred on 2 Oct, 2007 and α-amylase (545.3 KNU/ml; Eggleston et al, 2008) was added at 0 (control), 2, and 5 ppm on cane weight as a working solution (diluted 3-fold [1:2 dilution] in distilled water at the factory) into the inlet pipe of the next-to-the last evaporator (Figure 1a). Samples of syrup entering (before α-amylase was applied) and exiting the next-to-the-last A_2 evaporator were collected, as well as the syrup exiting the last A_3 evaporator, taking into account the 11 and 18 min R_ts of the next-to-the-last and last evaporators, respectively. Samples were collected six times every 20 min, and 1 hour was left between each dose level studied to equilibriate the system.

On the second day of sampling (3 Oct), the same α-amylase working solution (freshly prepared) was applied directly into the last A_3 evaporator only (Figure 1b). Samples of syrup entering and exiting the last evaporator were collected, taking into account the 18 min R_t. Samples were collected six times every 20 min, and

Figure 2a. Changes in maximum, minimum and average starch concentrations in the syrups entering the next-to-last (A_2) evaporator across the 2007 processing season. Sampling dates are noted on the figure

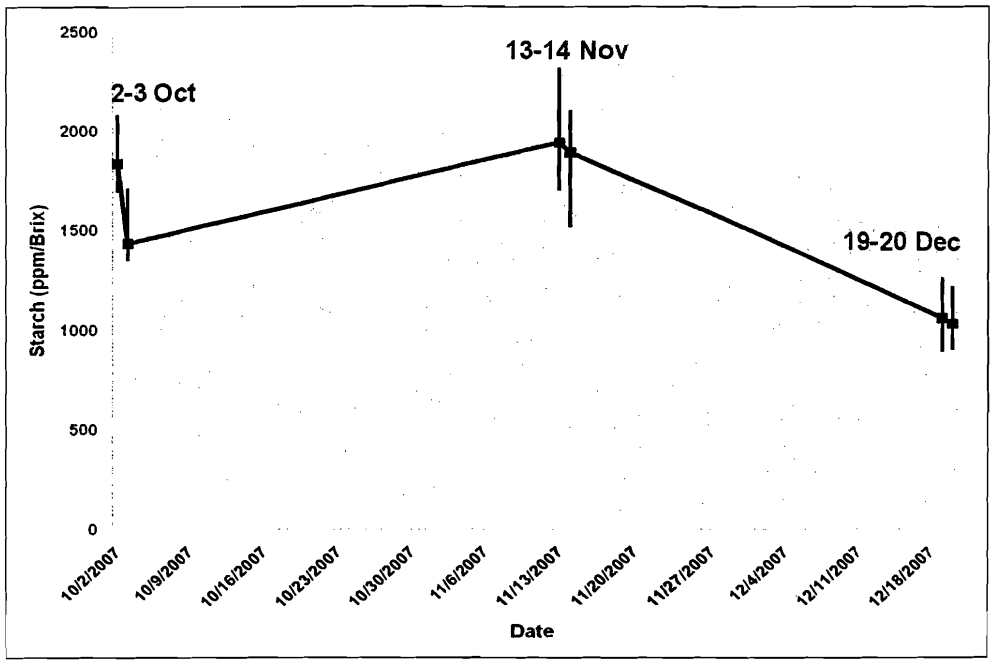
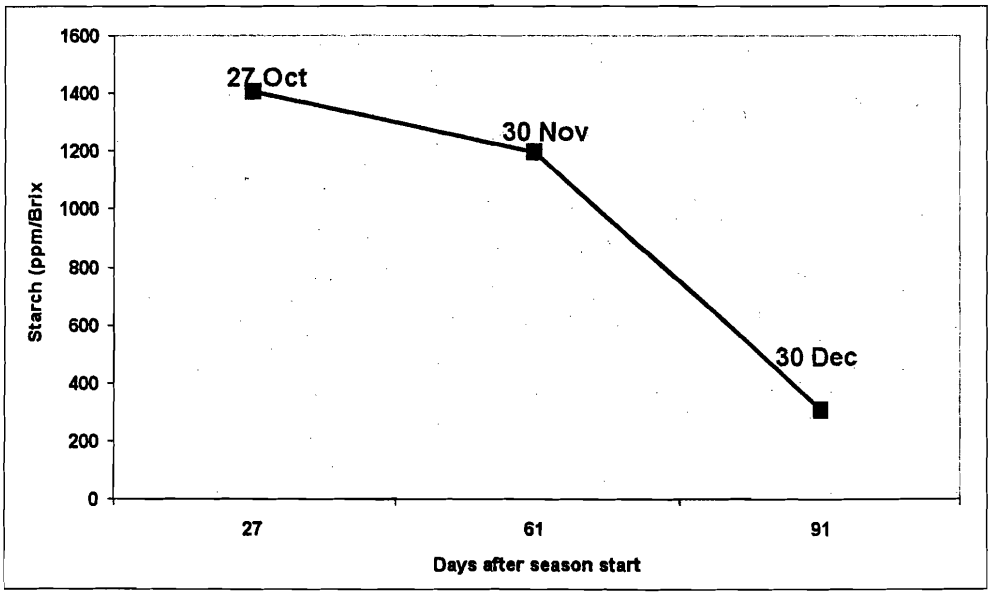


Figure 2b. Starch reduction in mixed juice at a Louisiana factory across the 1999 processing season. From Egglestone *et al* (2002). Sampling dates are noted on the figure



1 hour was left between each dose level studied to equilibriate the system.

The 2 day sampling period was repeated three times (Oct 2-3; Nov 13-14; Dec 19-20) across the 2007 LA processing season.

Statistics

Pearson correlation coefficients were calculated to investigate relationships among the different chemical and thermal parameters using Microsoft Excel 2007™. Mean separation of values were obtained using Duncan's New Multiple Range Test at the 5% probability level using PC-SAS (SAS Institute, Cary, NC).

Results and discussion

Starch concentrations across the processing season

Maximum, minimum, and average starch concentrations in syrups entering the next-to-the-last (A_2) evaporator, i.e., before α-amylase was applied, are illustrated in Figure 2a (see also Table 1). Although starch concentrations decreased significantly ($P<0.05$) from early to late season (av. 1760 ± 189 ppm/Brix in early Oct to av. 1055 ± 106 ppm/Brix in late Dec) they actually increased in mid-season (av. 1925 ± 190 ppm/Brix in mid Nov). Early season sugarcane is immature and contains more starch (a storage polysaccharide) and as it matures the plant utilizes the starch and the concentration decreases. However, the reduction of starch across the season varies among sugarcane varieties mostly because of their differing maturing characteristics. In previous years, Louisiana was highly dependent on one variety LCP 85-384 and starch delivered with LCP 85-384 decreased steadily across the season. This is illustrated in Figure 2b, which shows the reduction of starch in factory mixed juice across the 1999 LA processing season (Eggleston *et al*, 2002). However, since circa 2005 the yields of LCP 85-384 dramatically decreased because of severe susceptibility to brown rust disease, which caused the introduction of new sugarcane varieties with differing maturity characteristics. Eggleston *et al* (2010) recently reported that starch in sugarcane juice is now increasing until the end of Nov in Louisiana, before decreasing markedly in Dec, most because of HoCP 96-540 and L 99-233 two of the most popular new varieties. It is also of interest to compare the considerably higher starch concentrations in 1999 (Eggleston *et al*, 2002) compared to 2007 (compare Figure 2a with 2b), which not only illustrates the different varieties grown in Louisiana since 1998 but also the considerably higher amount of sugarcane trash, i.e., leaves and tops containing starch, that has been delivered to Louisiana factories.

The year-to-year changes in starch concentrations delivered with sugarcane to factories across the season (Figure 2) highlights (i) the need for processors to monitor starch across the season

and not just assume typical trends and (ii) for sugarcane breeders to consider processing implications of released varieties. Zhou *et al* (2008) showed that breeding for low concentrations in sugarcane was possible and that it could even increase valuable sucrose concentrations. Chemical glyphosate ripener is routinely applied to commercial LA sugarcane to accelerate the maturation process in early season and increase sucrose yields. The effect of glyphosate on starch concentrations, however, is dependent on the variety and can either increase or decrease on artificial ripening (Eggleston *et al*, 2007b).

Factory trials on α-amylase applications in next-to-the-last and last evaporator syrups

Due to the present concern of carry-over HT stable α-amylase activity in molasses and raw sugar (Eggleston *et al*, 2007a), this factory trial was only conducted with an intermediate temperature (IT) stable *B. subtilis* α-amylase. A commercial *B. subtilis* IT α-amylase with the highest available activity per unit cost (118.3 KNU/ml/$) was chosen for the trials. As we previously observed that a working solution of α-amylase increased the hydrolysis of starch in syrup at the factory because of improved contact between the starch and α-amylase (Eggleston *et al*, 2008), a working solution (1:2 dilution in water, prepared at the factory) of the enzyme was applied in these trials.

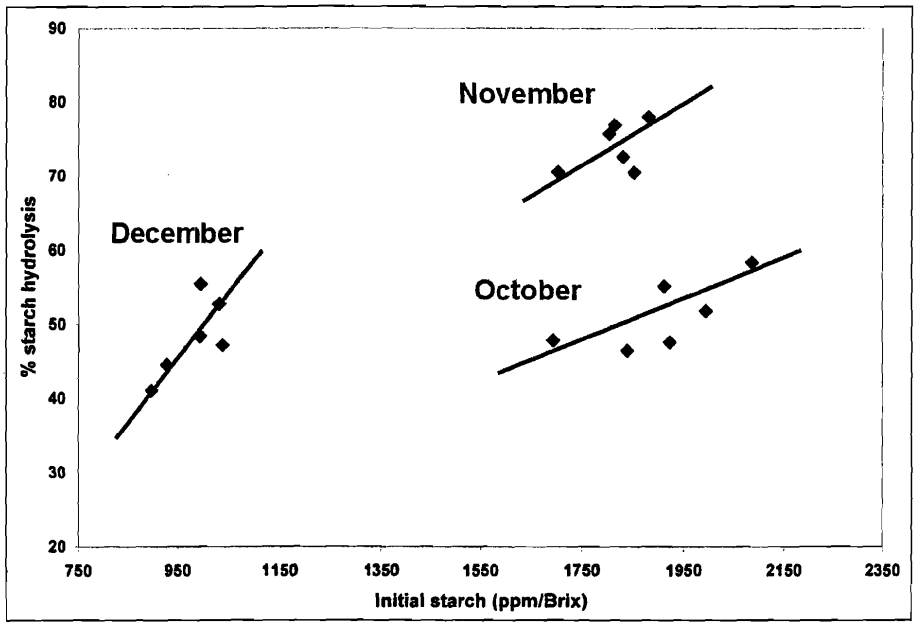
Results of the comparative effect of applying the α-amylase to either the next-to-the-last (A_2) or the last (A_3) evaporator at 0, 2, and 5 ppm/cane wt. dose levels across the season, are listed in Table 1. As expected, when α-amylase was applied to either evaporator, percent starch hydrolysis increased with increasing dose level which was significant at the 5% probability level. The hydrolysis increase was polynomial rather than linear at both the individual 2 or 5 ppm dose levels as well as when all the data was combined (not shown). This polynomial relationship indicates that

Table 1. The effect of the two different IT stable α-amylase treatments on starch concentrations and starch hydrolysis across the 2007 LA processing season (Oct to Dec)

Dose	Av. starch concentration ppm/Brix ± standard deviation*			Range of total starch hydrolysis †	Av. total starch hydrolysis
ppm/cane weight	A_2 Syrup$_{in}$	A_2 Syrup$_{out}$	A_3 Syrup$_{out}$	%	%[b]
AMYLASE ADDED TO THE NEXT-TO-THE-LAST (A_2) EVAPORATOR					
October 2					
0	1812.7 ± 51.7A‡	1763.1 ± 36.2A	1794.3 ± 46.2A	+7.6 to 4.1	**0.9 ± 4.4C**
2	1801.7 ± 68.4A	661.0 ± 528.5B	1134.9 ± 136.0Ba#	27.0 to 45.9	**37.0 ± 6.9B**
5	1907.1 ± 133.5A	217.6 ± 48.6Cb	926.4 ± 65.1Ca	46.5 to 58.4	**51.2 ± 4.8Aa**
November 13					
0	2267.5 ± 45.1A	2283.7 ± 10.4A	2379.5 ± 55.9A	+7.4 to +4.3	**+4.9 ± 1.7Ca**
2	1853.6 ± 77.6B	714.5 ± 334.9Bb	1042.6 ± 141.6Ba	31.5 to 52.8	**43.6 ± 8.3Ba**
5	1813.7 ± 60.9B	303.4 ± 11.9Cb	470.5 ± 53.9Cb	70.5 to 78.0	**74.0 ± 3.3Aa**
December 19					
0	1160.5 ± 84.7A	1149.4 ± 13.1A	1140.9 ± 23.7A	+9.0 to 12.0	**1.2 ± 9.0Ca**
2	1086.9 ± 86.3A	526.7 ± 219.3Bb	691.0 ± 38.7Ba	28.2 to 45.5	**36.1 ± 5.8Ba**
5	981.1 ± 56.8	303.2 ± 29.2Cb	505.8 ± 6.5Cb	41.1 to 55.5	**48.3 ± 5.3Aa**
AMYLASE ADDED TO THE LAST (A_3) EVAPORATOR					
October 3					
0		nd	nd	-	-
2		1433.2[d]	1001.2 ± 42.5Aa	nd	**30.1[d]**
5		1433.2 ± 156.0a	831.4 ± 68.4b	35.0 to 47.2	**41.7 ± 5.0b**
November 14					
0		2283.7 ±10.4A§	2379.5 ± 55.9A§	+7.2 to 0.7	**+3.8 ± 3.4Ca**
2		1883.5 ± 174.8Ba	973.7 ± 67.7Ba	37.8 to 51.5	**46.6 ± 5.3Ba**
5		2013.5 ± 77.8Ba	878.5 ± 38.1Ca	53.0 to 59.8	**56.3 ± 0.4Ab**
December 20					
0		1149.4 ± 13.1A"	1140.9 ± 23.7A"	+2.8 to 3.5	**0.7 ± 2.9Ba**
2		955.0 ± 49.8Ba	709.5 ± 46.3Ba	21.9 to 29.3	**25.7 ± 3.2Aa**
5		1100.8 ± 113.2Aa	705.3 ± 93.1Ba	21.1 to 50.5	**34.8 ± 14.5Aa**

* N=3-6
† Av % total starch hydrolysis was calculated as (starch A3out – starch A2in/starch A2in) x 100
‡ The same upper case letters represent no statistical difference (P<.05) among the three levels of doses on the same day only.
# The same lower case letters represent no statistical difference (P<.05) for the same sample type between the two treatment types
§ Estimated value
" Samples collected day before

Figure 3. Effect of initial starch concentration on the percent starch hydrolysis when α-amylase was added to the next-to-last (A_2) evaporator. α-Amylase dosage was 5 ppm/ton cane wt



it is difficult to predict how to adjust the α-amylase dosage for optimized starch hydrolysis in the factory.

Although among the three different dose levels, there were no significant differences for starch concentrations in the syrups entering the evaporator (A_{2in}), once α-amylase was applied into the next-to-the-last (A_2) evaporator, statistical differences ($P<.05$) did occur at all three dose levels for starch in syrups exiting the A_2 evaporator (A_{2out}). Moreover, these statistical differences were subsequently reflected in syrups exiting the last A_3 evaporator (Table 1). Therefore, significant ($P<.05$) hydrolysis of starch in syrups occurred when α-amylase was applied to the next-to-the-last evaporator which is further evidenced by the significantly different values for total starch hydrolysis at the three dose levels (up to 78.0% in Nov at the 5 ppm dose level; Table 1). Starch concentrations in the syrups exiting the A_2 evaporator, however, were always lower than those exiting the subsequent last (A_3) evaporator (Table 1). This suggests that the feed syrup (A_{2out}) into the last evaporator was still mixing with syrup containing higher starch concentrations already resident there. (There was no evidence of more starch hydrolysis in the last evaporator, although this was most likely was because of the 18 min R_t). Nevertheless, when α-amylase was applied into the next-to-the-last evaporator, more ($P<.05$) starch hydrolysis occurred across both A_2 and A_3 evaporators (from A_{2in} to A_{3out}) than when it was applied into the last evaporator alone (from A_{2out} to A_{3out}). Furthermore, this greater starch hydrolysis occurred for all three months studied, particularly at the 5 ppm dose level (Table 1). Lack of a significant difference between the two α-amylase applications in Dec, at both dose levels (Table 1), most likely reflects the difficulty in hydrolyzing low starch concentrations which is discussed further in the next section. Reasons for the better, overall starch hydrolysis in the next-to-the-last than the last evaporator are multi-fold: (i) the lower Brix levels in the next-to-the last evaporator are more optimum for α-amylase action, (ii) more water is available as a reactant for the hydrolysis reaction, and (iii) there is more time for the hydrolysis reaction to occur.

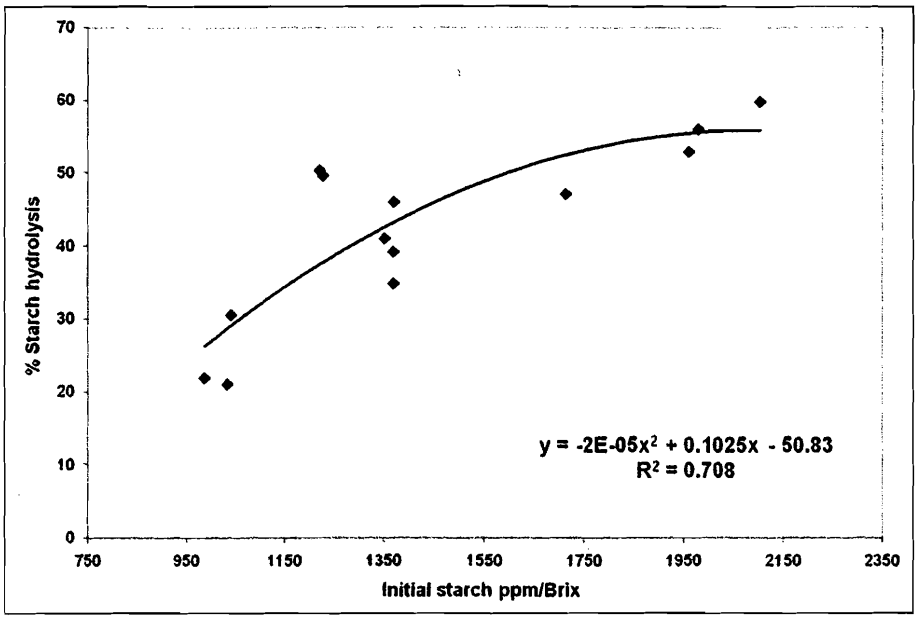
Standard deviations associated with total starch hydrolysis values in Table 1 indicate the precision of the α-amylase application to hydrolyze starch. Precision is important to engineers for process control purposes. Generally, when α-amylase was applied to either evaporator, the precision of starch hydrolysis was slightly worse at the 2 ppm than the 5 ppm dose level (Table 1 and Figure 2a), and this was more clearly evident when it was applied to the next-to-the-last evaporator. Furthermore, for both applications at the 5 ppm dose level, precision was best in mid-season (Nov) when starch concentrations were the highest (Table 1).

Overall, the greatest av. total starch hydrolysis that occurred was still only 78.0%. (the highest average value was 74.0%) (Table 1). Greater hydrolysis values may not have been obtained because of the limitations of the method used to measure starch concentrations. The method (Godshall *et al*, 2004) was iodometric, which is typical of sugar industry starch methods. For example, all ICUMSA (International Commission for Uniform Methods in Sugar Analysis) starch methods are iodometric. However, iodometric methods underestimate some starch hydrolysis because a negative starch value requires starch at ~DP 18 or lower (Bailey and Whelan, 1961). Furthermore, for complete hydrolysis of starch to glucose, glucoamylase must be added to hydrolyze (1→6) glycosidic bonds in the amylopectin molecule, although there are much less of these bonds compared to (1→4) bonds that α-amylase hydrolyzes. These two reasons, therefore, may explain at least partially why >74.0% average total hydrolysis was not achieved in this study.

How initial starch concentrations affect starch hydrolysis at the factory

The effect of initial starch concentrations in syrup on the percent starch hydrolysis across the season by α-amylase (5 ppm dose) in the next-to-the-last (A_2) evaporator is illustrated in Figure 3. In general, as the initial starch concentration increased, the greater was the starch hydrolysis (Figure 3) with better precision (Table 1). However, this relationship was only moderately strong and highly dependent on the seasonal variation in starch concentrations, particularly in the syrups entering the A_2 evaporator. Polynomial curve fits were, generally, better than linear curve fits, and three distinct curves occurred in Figure 3 each representing a different month of study. When the data at the 2 ppm dose level was combined with that at the 5 ppm level these three distinct curves still existed (not shown). The weaker relationship in Oct (Figure 3) may be linked with the occurrence of the highest range of starch concentrations in that month and possibly the occurrence of impurities inhibiting α-amylase action. The worst quality sugarcane is typically delivered to the factory in Oct, i.e., early

Figure 4. Effect of initial starch concentration on the percent starch hydrolysis when α-amylase was added to the last (A_3) evaporator alone. α-Amylase dosage was 5 ppm/ton cane wt



season (Eggleston *et al*, 2010).

The effect of initial starch concentrations in syrup on the percent starch hydrolysis by α-amylase (5 ppm dose) in the last (A3) evaporator alone is illustrated in Figure 4. Although as the initial starch concentration increased the greater was the starch hydrolysis, this relationship was only moderately strong (Figure 4) and only slightly stronger at the 2 ppm dose level (not shown). In comparison to the application of α-amylase into the next-to-the-last evaporator (Figure 3), there was no clear distinction between the three different months. This suggests that seasonal variations in starch have less detrimental effect on α-amylase application in the last evaporator or it may just reflect the mixing of syrups entering and already residing in the last evaporator that have varying starch concentrations. Nevertheless, less starch hydrolysis occurred when α-amylase was applied to the last evaporator compared to application in the next-to-the-last evaporator (Table 1).

Overall, these results strongly suggest it is usually easier to hydrolyze starch with α-amylase at the factory when starch concentrations in syrup are relatively high, i.e, >~1000 ppm/Brix. This can be attributed to the higher contact between the starch (substrate) and α-amylase (enzyme). This also may explain why, for both locations of α-amylase application at the 5 ppm dose level, precision was best in mid-season (Nov) when starch concentrations were the highest (Table 1).

Conclusions and future work

Factory trials were conducted across the 2007 Louisiana processing season (Oct-Dec) to optimize α-amylase application across varying environmental conditions and physiological states of sugarcane. Application of a working solution (diluted 3-fold in tap water at the factory) of IT stable α-amylase of high activity (545.3 KNU/ml) and high activity per unit cost (118.3 KNU/ml/$) to the next-to-the-last evaporator provided significantly (P<.05) greater starch hydrolysis (up to 78.0% at a 5 ppm/cane wt dose) than applying it to the last evaporator alone (only up to 59.8% at a 5 ppm/cane wt dose). Reasons for the improved starch hydrolysis in the next-to-the-last than the last evaporator are multi-fold: (i) the lower Brix levels in the next-to-the last evaporator improve α-amylase action, (ii) more water is available as a reactant for the hydrolysis reaction, and (iii) there is more time for the hydrolysis reaction to occur. Significantly (P<.05) less starch was hydrolyzed with lower precision when starch concentrations were low, i.e., <1000 ppm/Brix, because of lower contact between the starch (substrate) and α-amylase (enzyme). Changing starch concentrations across the season make standardized application of α-amylase impossible. Application doses will have to vary across the season for maximum optimization which, in turn, will only occur if starch concentrations are monitored consistently.

Problems associated with starch in sugarcane have traditionally been alleviated through the application of α-amylase during processing mostly because of the need to minimize the number of selection criteria in sugarcane breeding programs (Zhou *et al*, 2008). This may have worked in some countries but is not universally feasible or, as shown in this study, is not entirely efficient and economical in sub-tropical areas where immature sugarcane is harvested. Moreover, α-amylases only hydrolyze starch into dextrin oligosaccharides (maltoses) that could be detrimental to crystallization and filterability of raw sugars. The widespread adoption of billeted and green (unburnt) sugarcane harvesting techniques has exacerbated the problem because starch is higher in green leaves and tops. Newer sugarcane varieties with higher starch concentrations for a longer period of time have also contributed to the problem (Eggleston *et al*, 2010). It may be possible to bio-engineer an IT stable α-amylase to be more active in high Brix syrups, but this is extremely expensive and such an investment by a large enzyme company is not foreseen as the sugar market is viewed as being too small for α-amylase (Eggleston *et al*, 2008). In the near-term, optimization studies to improve the seasonal operating conditions of α-amylases as outlined in this paper are the best solutions. The use of uniform ultrasound technology (Yachmenev *et al*, 2007) to improve contact between the α-amylase and starch could only enhance industrial optimization. However, breeding for sugarcane varieties that accumulate low levels of starch likely will alleviate the problem and provide a more preventable, economical and sustainable long-term solution. Zhou *et al* (2008) recently reported that it is possible to breed for low starch varieties that could have the great added attraction, to both sugarcane growers and processers, of increased sucrose yields as well. Furthermore, low starch varieties consistently produced lower and more stable starch across replications, years and locations compared to high starch varieties (Zhou *et al*, 2008).

Recommendations for α-amylase factory applications during the processing season

From the above trials and other studies (Eggleston *et al*, 2007a, 2008) we put forward the following recommendations:

• Measure the activity of commercial α-amylases at your factory to (i) compare the economically equivalent activities of different commercial amylases, (ii) monitor the changing activities of α-amylases on factory storage, and (iii) measure the activity of delivered batches.

* Ask vendors from which *Bacillus* source the amylase comes from. Ensure it is an intermediate temperature stable α-amylase from *Bacillus subtilis*.

• Addition of high activity α-amylase as a working solution (prepared with tap water at the factory) is more economical than adding undiluted or low activity α-amylase.

* To prepare the working solution mix 1:3 with tap water. The working solution is storable for up to approximately 12 hours at the factory.

• Add α-amylase to syrup in the next-to-the last evaporator.

* Ensure it is added directly into the next-to-the last evaporator and not the inlet pipe.

• Recommended dosage of a working solution of high activity *B. subtilis* IT amylase (545 KNU/ml) is 5ppm/cane wt.

* Do not decrease the α-amylase dosage in processing season when starch concentrations entering the factory are lower. This is because relatively lower concentrations of starch are more difficult to break down by α-amylase due to the lower contact between the α-amylase and the starch.

* When considerably lower starch concentrations occur in the processing season, i.e., less than 1000 ppm/Brix, they may not be causing any processing problems. Factory processors need to decide whether to discontinue α-amylase addition altogether when low concentrations of starch are being delivered to the factory, to save costs.

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